The Journal of Microbiology, December 2003, p.340-344 Vol. 41, No. 4

NOTE

Replacement of Thymidine Phosphorylase RNA with Group I Intron of *Tetrahymena thermophila* by Targeted *Trans*-Splicing

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(Received August 18, 2003 / Accepted October 28, 2003)

The group I intron from *Tetrahymena thermophila* has been demonstrated to employ splicing reactions with its substrate RNA in the *trans* configuration. Moreover, we have recently shown that the *trans*-splicing group I ribozyme can replace HCV-specific transcripts with a new RNA that exerts anti-viral activity. In this study, we explored the potential use of RNA replacement for cancer treatment by developing *trans*-splicing group I ribozymes, which could replace tumor-associated RNAs with the RNA sequence attached to the 3′ end of the ribozymes. Thymidine phosphorylase (TP) RNA was chosen as a target RNA because it is known as a valid cancer prognostic factor. By performing an RNA mapping strategy that is based on a *trans*-splicing ribozyme library, we first determined which regions of the TP RNA are accessible to ribozymes, and found that the leader sequences upstream of the AUG start codon appeared to be particularly accessible. Next, we assessed the ribozyme activities by comparing *trans*-splicing activities of several ribozymes that targeted different regions of the TP RNA. This assessment was performed to verify if the target site predicted to be accessible is truly the most accessible. The ribozyme that could target the most accessible site, identified by mapping studies, was the most active with high fidelity *in vitro*. Moreover, the specific *trans*-splicing ribozyme reacted with and altered the TP transcripts by transferring an intended 3′ exon tag sequence onto the targeted TP RNA in mammalian cells with high fidelity. These results suggest that the *Tetrahymena* ribozyme can be utilized to replace TP RNAs in tumors with a new RNA harboring anti-cancer activity, which would revert the malignant phenotype.

***Key words*:** group I intron, ribozyme, RNA replacement, *Tetrahymena thermophila*, thymidine phosphorylase, *trans*-splicing

The group I intron from *Tetrahymena thermophila* is a ribozyme that performs two *trans*-esterification reactions in order to excise itself from a precursor transcript (Cech, 1993). A derivative of the self-splicing intron, known as L-21 (lacking the first 21 nucleotides of the intron), was shown to *trans*-splice an exon tagged at its 3′ end onto a separate RNA in mammalian cells, as well as in bacteria. This splicing occurred after the recognition of the target RNA by base pairing to any accessible uridine nucleotides of the RNA, which was directed by the internal guide sequence (IGS) of the ribozyme (Sullenger and Cech, 1994; Jones *et al*., 1996). With these targeted *trans*-splicing reactions, the ribozyme was shown to revise mutant transcripts associated with several human genetic and malignant diseases (Lan *et al*, 1998; Phylactou *et al*., 1998; Watanabe and Sullenger, 2000; Rogers *et al*., 2002; Shin *et al*., 2002). Moreover, we recently demonstrated that the *trans*-splicing ribozymes could selectively induce any therapeutic gene activities in HCV RNA-expressing cells via the specific RNA replacement of the HCV RNA (Ryu *et al*., 2003), which implies that *trans*-splicing ribozymes could be potentially used for the treatment of a wide range of human diseases, such as cancer, through the RNA replacement of the disease-associated unique RNAs.

Thymidine phosphorylase (TP), an enzyme that is involved in the reversible phosphorylation of thymidine, deoxyuridine, and their analogues to their respective bases and 2-deoxy-D-ribose-1-phosphate, is identical to the platelet-derived endothelial cell growth factor (PD-ECGF) (Iltzsch *et al*., 1985; Furukawa *et al*., 1992). TP stimulates chemotaxis and the [^{3}H] thymidine incorporation by endothelial cells *in vitro*, and has angiogenic activity *in vivo* (Miyazono *et al*., 1987; Miyadera *et al*., 1995; Brown *et al*., 2000). Compared with adjacent non-neoplastic tissues, TP is expressed at much higher levels in a wide vari-

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ety of tumors (Luccioni *et al*., 1994; Fox *et al*., 1995; Takebayashi *et al*., 1996). In addition, TP enhances tumor progression and confers resistance to apoptotic signal pathways (Ikeda *et al*., 2002; 2003; Mori *et al*., 2002). Therefore, TP is a valid hallmark of cancer and an important target for cancer therapies.

Here, in order to develop a therapeutic approach to TP-associated malignant disease, we tested whether group I-based ribozymes could be utilized to replace the TP RNA with the intended sequences attached to their 3′ end by targeted *trans*-splicing. To this effect, we first identified the sites of TP RNA that were most accessible to the ribozymes. We then constructed specific ribozymes that targeted those sites and determined if the ribozymes could efficiently and correctly *trans*-splice the target RNA not only *in vitro* but also in mammalian cells.

Every uridine in TP RNA can be potentially targeted by group I ribozyme of *Tetrahymena thermophila* through G-U base pairing between the IGS of the ribozyme and the target RNA. However, only a limited number of uridines on the substrate RNA could be actually accessible to the ribozyme due to the target RNA′s tertiary structure and/or complex formation with proteins in cellular circumstances (Lan *et al*., 2000). Thus, an RNA mapping strategy was first carried out to determine which uridines in the TP RNA are accessible to ribozymes. The mapping method was based on a *trans*-splicing ribozyme library (Lan *et al*., 1998; 2000; Ryu *et al*., 2003) and RNA tagging (Jones *et al*., 1996) as shown in Fig. 1. The ribozyme library was constructed by randomizing the IGS of the *Tetrahymena* group I intron so that the 5′ end of the ribozyme in the library began with 5′-GNNNNN-3′, where G represents guanine and N represents equimolar amounts of the four nucleotides (nt), and called GN5 ribozyme library (Lan *et al*., 1998). Thus, ribozymes in the GN5 ribozyme library would target and cleave the substrate RNA at any accessible uridine (U) residue and splice their 3′ exon to the 3′ end of 5′ cleavage target transcript. Part of the *lacZ* gene was utilized as a 3′ tagging exon in the GN5 ribozyme library that can be transferred onto the target RNA′s Us accessible to the ribozyme. The substrate TP RNA was generated by *in vitro* transcription using T7 RNA polymerase with a cDNA clone of TP (Ishikawa *et al*., 1989; a kind gift from C.-H. Heldin, Ludwig Institute, Sweden). To map the TP RNA, the GN5 ribozyme library (10 nM) was reacted at 37ºC for 3 h under splicing conditions (50 mM HEPES, pH 7.0, 150 mM NaCl, 5 mM $MgCl_2$) in the presence of a guanosine (100 μM) with the TP RNA (200 nM). The resulting *trans*-splicing reaction products were then amplified by RT-PCR with a 3′ tag primer specific for the ribozyme′s 3′ exon *lacZ* sequence (5′-ATGTGCTGCAAGGCGATT-3′) and a 5′ primer encompassing the 5′ end of target TP RNA (5′-CCGGAATTCTAATACGACTCACTATAGGGCAGTGGACCGCTGTG-3′) (Jones *et al*., 1996). The amplified products were cloned into several pUC19 vectors. Sequence analyses of the splicing junction sites showed that several uridines, which were present in the leader sequence upstream of the AUG start codon (position 124 nt), appeared to be particularly accessible because more than 50% of the reaction products resulted from splicing at these sites (Fig. 2). In particular, the most accessible site was present in the uridine at position 73 (U73) on TP RNA. Interestingly, U73 was located in the loop part on the TP RNA secondary structure that was expected to be accessible to ribozyme.

Even though U73 could be predicted as a ribozyme accessible site from structure prediction, other uridines on the loop region of TP RNA, such as uridine at position

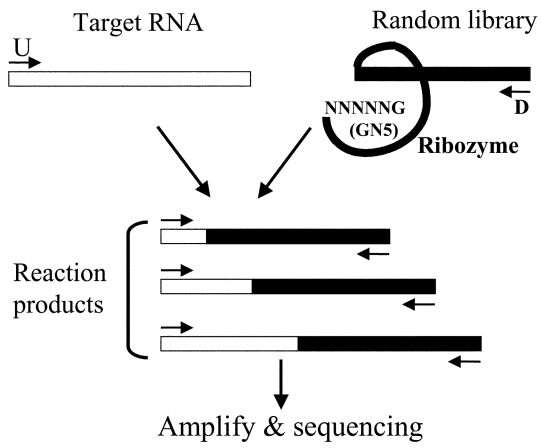

Fig. 1. Scheme for mapping accessible sites in TP RNA *in vitro* with a *trans*-splicing ribozyme library. The GN5 ribozyme library and target TP transcript were incubated in the reaction buffer at 37 ºC for 3 h in the presence of a guanosine. Reaction products were amplified with a 5′ primer specific for the TP RNA and with a 3′ primer recognizing 3′ exon tag *lacZ* sequence, were cloned, and sequenced.

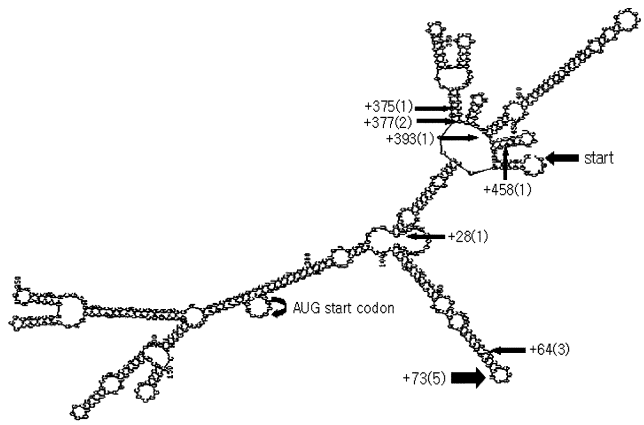

Fig. 2. Mapping results of the ribozyme-accessible sites in TP RNA. Nucleotide positions of the accessible uridines identified from *in vitro* mapping analysis are indicated by arrows as nucleotide numbers on the secondary structure of TP RNA predicted by the computer simulating program called mfold (Jager *et al*., 1989). The number of clones containing a given uridine at the splice site is presented in parentheses. AUG residue located at nt 124-126 denotes the initiator codon of the TP protein.

107 (U107), would be also expected to be possibly targeted by ribozymes. Thus, to verify if the sites predicted to be accessible by mapping studies are truly the most accessible sites to ribozymes, we assessed *in vitro trans*-splicing activities of two different ribozymes targeting uridines at position 64 (U64), or 73 (U73) in TP RNA, which were identified via mapping analysis, or two ribozymes targeting uridine at position 107 (U107), or 137 (U137), which were not detected from our mapping study (Fig. 3A). U64 and U137 were chosen because they are present in the stem region of the TP RNA that could be expected to be less accessed to ribozymes (Fig. 2). Rib64, Rib73, Rib107, or Rib137 ribozymes recognizing these individual sites were generated by *in vitro* transcription of DNA templates, which were created from pT7L-21 by PCR with a 5′ primer containing the T7 promoter and each ribozyme′s IGS and also with a 3′ primer specific for the 3′ exon *lacZ* sequence. The pT7L-21 vector, which was kindly supplied by B.A. Sullenger at Duke University, encodes a slightly shortened version of the natural group I intron from *Tetrahymena*, called L-21 (Sullenger and Cech, 1994). The IGS on the L-21 *trans*-splicing ribozyme (5′-GGAGGG-3′) was exchanged with 5′-GCCCGG-3′ in Rib64, 5′-GGCCCA-3′ in Rib73, 5′-GGGGCG-3′ in Rib107, or 5′-GUCAAG-3′ in Rib137. In addition, inactive ribozymes, R(d)64, R(d)73, R(d)107, or R(d)137, which are devoid of the catalytic core of the enzyme (Sullenger and Cech, 1994), were constructed as negative controls. The specific ribozymes (100 nM) were incubated under splicing conditions with the target TP RNA (10 nM). RT-PCR analyses were then performed with a 3′ primer specific for the 3′ exon tag sequence and a 5′ primer specific for TP RNA as described. An amplified fragment of the expected size of 93 bp, 102 bp, 136 bp, or 166 bp was generated from the reaction mixtures with the TP RNA and Rib64, Rib73, Rib107, or Rib137, respectively (Fig. 3A, lanes 3-6). It should be noted that Rib73 can *trans*-splice a 3′ exon tag onto the TP RNA with the highest efficiency. Moreover, although U64 is present on the stem part of the RNA, Rib64 can react with the target RNA with the second most efficiency. However, much less RT-PCR products were detected from samples with both Rib107 and Rib137, indicating that U107 and U137 in the TP RNA were less accessible to the ribozymes. Thus, these results strongly indicate that the relative *trans*-splicing efficiency at the chosen sites corresponds with the predicted accessibility from our mapping analyses, but not with the predicted RNA secondary structure. The inactive versions of all four ribozymes in the reaction with the target RNA, substrate RNA alone, or Rib73 alone could not produce any *trans*-splicing products (Fig. 3A, lanes 7-12). These results suggest that the amplified RT-PCR products found in lanes 3-6 of Fig. 3A resulted from the catalytic activity of the ribozymes. To determine if the specific ribozyme could accurately perform the *trans*-splicing reaction with the target RNA, sequence analyses of the amplified spliced products found in lane 4 of Fig. 3A were carried out. The sequence of the 102 bp RT-PCR fragment demonstrated that the ribozyme, Rib73, had correctly targeted the TP RNA at the predicted reaction site (U73) and replaced sequences downstream of the reaction sites with the 3′ exon sequences attached to the 3′ end of the ribozyme (Fig. 3B). Sequencing analyses of reaction products isolated from lane 3, 5, or 6 of Fig. 3A demonstrated that Rib64, Rib107, or Rib137 also correctly *trans*-spliced its 3′ exon tag onto the TP target RNA at the predicted reaction site (data not shown). From these results, it was concluded that specific ribozymes that target the predicted accessible sites could replace TP RNA with a 3′ exon tagged at the 3′ end of the ribozymes by targeted *trans*-splicing with high fidelity. *Trans*-splicing analyses, taken together with the mapping studies, encouraged us to focus on further studies with Rib73.

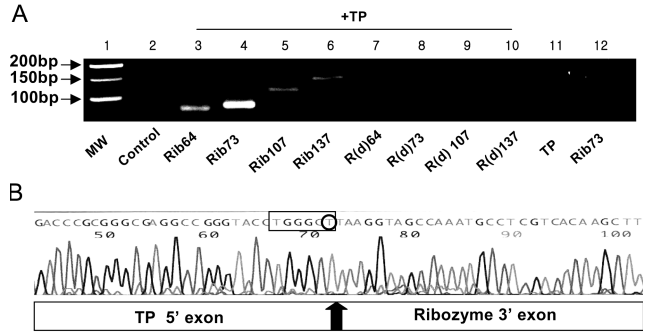

Fig. 3. Analysis of *trans*-spliced products *in vitro*. (A) RT-PCR analysis of *trans*-spliced RNA products generated *in vitro*. A series of active (lanes 3-6) or inactive ribozymes (lanes 7-10) were incubated with TP target RNA substrate, and *trans*-spliced products were amplified. As a reaction control, RT-PCR products without any RNA (lane 2), with TP RNA alone (lane 11), or Rib73 alone (lane 12) were presented. Amplification products are then subjected to electrophoresis in a 3% agarose gel. The migration of 50 bp ladder is indicated as a molecular mass marker (lane 1, MW). (B) Sequence analysis of *trans*-splicing products produced *in vitro*. The amplified products from *trans*-splicing reaction between Rib73 and TP RNA (Fig. 3A, lane 4) were isolated on a gel and cloned. Sequence of one representative clone out of 10 different clones with same sequence is shown. The expected sequence around the splicing junction, indicated by the arrow, was shown with the ribozyme recognition sequence in TP RNA (boxed) and the uridine at position 73 (circled).

To determine whether Rib73 could *trans*-splice TP RNA at the predicted U73 with fidelity in cells, NIH3T3 cells were either mock-transfected or cotransfected with 1 μg of TP RNA with or without 4 μg of Rib73 or R(d)73 using 3 μl of DMRIE-C (Fig. 4). RNAs used for transfection into cells were modified at its 3′ end by adding poly(A) through the use of poly(A) polymerase at 37ºC for 20 min. The entire RNA was isolated from the cells 24 h after transfection with guanidine isothiocyanate (Feramisco *et al.*, 1982) supplemented with 20 mM EDTA. The RNA (5 μg) was reverse transcribed, in the presence

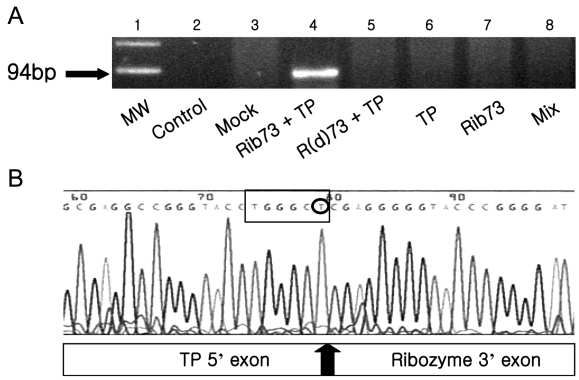

Fig. 4. *Trans*-splicing reaction with TP RNA in cells. (A) RT-PCR analysis of *trans*-spliced transcript generated in cells. NIH-3T3 cells were mock transfected (lane 3), transfected with TP RNA (lanes 4-6 and 8) alone (lane 6), with the active ribozyme (Rib73; lanes 4, 7 and 8), or with the inactive ribozyme RNA (R(d)73; lane 5). *Trans*-spliced products were amplified by RT-PCR, yielding a DNA fragment of 94 bp. (B) Sequence analysis of *trans*-spliced RNA products in cells. The amplified products in lane 4 of Fig. 4A were sequenced. Sequence of one representative clone of ten different clones with the same sequence is shown. The correct splicing junction is indicated with an arrow along with the ribozyme recognition sequence (boxed) and the nucleotide at the position 73 (circled).

of 10 mM L-argininamide, with a primer specific for the 3' tagging *lacZ* sequence. The resulting cDNAs were amplified for 40 cycles with a 5' primer specific for the *trans*-splicing junction (5'-CCGGAATTCCCGGGTAC-CTGGGCTCGAG-3') and with a 3' primer specific for the 3' exon *lacZ* sequence. A *trans*-spliced product of expected size (94 bp) was detected only in cells cotransfected with the ribozyme and the TP target RNA (Fig. 4A, lane 4). By contrast, no such product was found in cells mock-transfected, in those transfected with the target RNA alone, or in those transfected with the ribozyme alone (Fig. 4A, lanes 3, 6, and 7). Also, no product was generated in cells cotransfected with the inactive ribozyme (R(d)73) and the target RNA (Fig. 4A, lane 5). Moreover, no amplification product was detected from a "mix" RNA sample that was extracted after lysate from ribozyme-transfected cells was mixed with lysate from TP RNA-transfected cells. This suggests that the observed *trans*-splicing product was generated inside the NIH3T3 cells, but not during the RNA manipulation (Fig. 4A, lane 8). Furthermore, sequence analysis of the 94 bp amplified fragment, isolated from the lane 4 of Fig. 4A, demonstrated that the ribozyme Rib73 had correctly spliced its 3' exon tag onto the targeted U73 of the TP RNA in cells (Fig. 4B). Thus, these results indicated that, also in mammalian cells, the specific ribozyme was able to replace the TP target RNA with a 3' exon tagged at the 3' end of the ribozyme with high fidelity.

In this study, group I intron ribozyme from *Tetrahymena thermophila* has been developed to specifically replace, by targeted *trans*-splicing, the TP RNA with the intended sequence attached to the 3' end of the ribozyme in cells as well as *in vitro*. The 3' exon in the *trans*-splicing reaction can have virtually any RNA sequence changed (Sullenger and Cech, 1995), which implies that the new RNAs that exert anti-cancer therapeutic activity can be triggered selectively in TP-expressing cancer cells via RNA replacement, if they are attached to the ribozyme backbone that contained IGS identified here. Therefore, the ribozyme backbone developed in this study can be utilized for the development of modalities for specific gene therapy against TP-associated cancer. For example, the ribozyme could be modified to contain cytotoxin RNA sequence in the place of the *lacZ* sequence at the 3' end of the ribozyme. The ribozyme could then selectively induce cytotoxin activity in cancer cells expressing TP RNA, resulting in the specific elimination of the cancer cells. This RNA replacement would be a more attractive approach for cancer therapy because it should inhibit or reduce the production of the TP protein and simultaneously engender the production of therapeutic gene activity specifically in the TP-associated cancer cells. Furthermore, other RNA-associated malignant or infectious diseases would be also treated by *trans*-splicing ribozyme that could selectively replace the disease-associated specific RNAs with transcripts expressing therapeutic activity. Moreover, with RNA mapping studies, we identified uridines in the TP RNA that were most accessible to the ribozymes. Comparative *trans*-splicing analyses clearly showed that the ribozymes recognizing these isolated accessible sites are truly the most active. Recently, many anti-cancer or anti-viral protocols have been proposed based on inhibitory RNA or short oligonucleotides such as *trans*-cleavage ribozyme, *trans*-splicing ribozyme, antisense oligonucleotides, or siRNA (Sullenger and Gilboa, 2002). A key factor for the success of such RNA-based disease suppression is to identify the most accessible sites in the substrate RNA to the inhibitory RNA. Therefore, mapping methods developed in this study could be very useful ways to find sites efficiently targetable to the various inhibitory RNAs. In addition, the mapping analyses, as conducted here, could be exploited to also identify the most accessible sites in other tumor-associated unique RNAs or viral RNAs for the therapeutic modulation of various malignant or infectious diseases.

The present research was conducted by the research fund of Dankook University in 2002.

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